What makes Bright a proto-oncogene?

Shawn Mathur

Honors Thesis
Dean's Scholars Honors Program
Department of Biological Sciences

The University of Texas at Austin
May 12, 2009

_____
Philip W. Tucker, Ph.D. Molecular Genetics and Microbiology
Supervising Professor

_____
Ruth Buskirk, Ph.D. Molecular Genetics and Microbiology
Department of Biological Sciences Honors Advisor

Abstract

Cancer is now the second leading cause of death in the developed world and accounts for 1 out of every 4 deaths in the US (American Cancer Society). The fundamental defect of cancer is unregulated cell division. One way this can happen is from an increase in the activity of proteins which signal cell growth. These proteins, called proto-oncogenes, possess normal functions in cells but can induce unregulated cell division when permanently activated by mutation. Understanding the molecular biology behind proto-oncogenes may lead to insight into how cancer develops and aid in the development of better treatment options for cancer patients. We are interested in studying Bright, a proto-oncogene expressed in mouse B-cells, white blood cells which are an essential part of the immune system. Bright can induce proliferation when overexpressed in normal cells. Our ultimate goal is to understand the mechanism by which Bright causes cells to become cancer-like.

Bright's oncogenic activity is correlated with an increase in function of E2F1, a protein considered to be a master regulator of cell division. E2F1 controls the transcription of many genes needed to drive the cell cycle. Specifically, we are interested in understanding how Bright upregulates the ability of E2F1 to activate transcription of its target genes. Various lines of evidence suggest that Bright may disrupt the action of pRb/E2F1 complexes which normally repress E2F1 production at the E2F1 promoter. Several biological studies have been performed to investigate this possibility and to learn more about the action of Bright. In our first experiment, we determine which properties of Bright are necessary to transform cells. Second, we show that Bright can associate with pRb, suggesting that Bright may play a role in E2F1 regulation. Finally, chromatin immunoprecipitation assays suggest that Bright associates with the E2F1 promoter and may indeed affect the function of pRb/E2F1 repressive complexes. Thus, we provide evidence in support of a novel model for

Bright's oncogenic properties. Our findings are particularly relevant for patients with Activated B cell-like Diffuse Large B-cell Lymphoma, a highly pernicious cancer that appears to involve the human version of Bright. Further studies to explore the nature of Bright's interaction with the Rb/E2F1 protein complex may lead to a better prognosis for these patients.

Contents

Background

As cancer now becomes one of the leading causes of death in the developed world, research aimed at understanding the mechanism of cancer formation is becoming especially important. The work presented in this thesis is part of an ongoing project to understand what makes Bright, a transcription factor expressed in B-cells, a proto-oncogene. Proto-oncogenes are proteins which can induce unregulated cell division when they are overexpressed or permanently activated by mutation (reviewed in Darnell, 2002). In light of previous work, it is apparent that our simple question has a relatively complex answer. Peeper et al (2002) found that hDril1, the human orthologue of Bright, can cause uncontrolled cell division, a molecular hallmark of cancer, by interfering with an important tumor suppressor pathway. Does Bright itself possess the same oncogenic properties as hDril1? If so, what specific component(s) of the tumor suppressor pathway does Bright interfere with? These are our specific questions of interest. To address the first question, we have conducted senescence rescue assays to test if Bright indeed has oncogenic activity. To address the second question, we examine Bright's interaction with E2F1, a downstream target of the tumor suppressor pathway and an important transcription factor controlling G1/S cell cycle progression (reviewed in Dyson, 1998). Our results suggest that Bright is a proto-oncogene, like hDril1, and transforms cells by de-repressing E2F1.

The goal of this background section is to highlight concepts relevant to the story of Bright. The first two sections provide a summary of basic cancer biology and Ras induced senescence. These concepts are foundational to the experiments presented in the results section. The third and fourth sections introduce our protein of interest, Bright, and characterize the oncogenic properties of hDril1, the human version of Bright. Finally, the last section describes the mechanism of E2F1 regulation.

Cell Malignancy

Cancer, or uncontrolled cell proliferation, is essentially a disease of genetic mutation. Alterations in the sequence of genes can disrupt complex signaling pathways designed to regulate when a cell grows or divides (reviewed in Vogelstein and Kinzler, 2004; Hahn and Weinberg, 2002). The growth of cells is normally tightly regulated. In fact, almost all diploid mammalian cells are "programmed" to have a specific life span (Hayflick, 1995). Human embryo fibroblast cells can only live for 50-60 population doublings before undergoing natural growth arrest, or senescence. Essentially, senescent cells are "stuck" in the G0 phase of the cell cycle. Cessation of cell division is a natural phenomena caused by a number of factors including DNA damage, oxidative stress, increased activity of growth-suppressing proteins and telomere shortening (Serrano and Blasco, 2001). However, it is also possible for cells to become senescent through aberrant expression of oncogenic proteins. This process is highly abnormal and often is the basis for cancer formation. One way this can happen is through an activating mutation in a proto-oncogene, such as Ras or Myc (Campisi, 2001). Since proto-oncogenes normally function to promote cell growth, they are typically activators of cell division, pro-growth signaling molecules and transcription factors (Evan et al, 2001). An activating mutation in a proto-oncogene results in constitutive pro-growth signaling in the cell. These signals drive cell cycle progression and result in unregulated cell division. However, cells have developed a strategy to control inappropriate pro-growth signals. Tumor suppressor genes serve to slow down cell cycle progression. Since tumor suppressor genes possess anti-proliferation activity, they usually encode for regulators of the cell cycle, signals for DNA damage repair or inducers of normal cell death, called apoptosis (Evan et al, 2001). If both diploid copies of a proto-oncogene become activated and tumor suppressor genes lose function, cell growth becomes unregulated. A once normal eukaryotic cell will now transform into a cancer-like cell. Instead of undergoing senescence, transformed cells

become immortal and show decreased requirements for growth signals, resistance to apoptosis and loss of cell cycle control (Hanahan and Weinberg, 2000). The relationship between tumor suppressor genes and proto-oncogenes may become clearer by drawing an analogy to parts of a car. Tumor suppressor genes are analogous to the brakes, acting to slow down cell growth while proto-oncogenes are analogous to the gas-pedal, acting to speed up cell growth. When both the brakes stop working (inactivation of tumor suppressor genes) and the gas-pedal is stuck down (activation of proto-oncogenes), the car cannot be controlled (unregulated cell division).

It is now generally believed that cancer arises from multiple mutations in the cell. These mutations affect tumor suppressor genes and proto-oncogenes. This idea was first proposed by Carl Nordling in 1953 and then extended by Alfred Knudson in 1971. Knudson showed that the occurrence of retinoblastoma resulted from two independent mutations, each affecting one of the two retinoblastoma alleles (Knudson, 1971). Currently, the paradigm in cancer biology is that mutations in tumor suppressor genes and proto-oncogenes are both needed for cancer formation. Accordingly, a good way to test for oncogenic properties is to co-express a protein of interest with another known oncogene (assuming the protein of interest is suspected to affect the tumor suppressor pathway). This simulates two mutations in a cell: activation of an oncogene and inactivation of the tumor suppressor pathway. This is the theory behind "BTR cells", the model cell line which we use to study Bright.

Ras^{V12} induced senescence

Normally, the growth and proliferation of cells is tightly controlled by complex signaling pathways. The cell growth signal pathway begins with plasma membrane receptors binding growth factors outside the cell (Howe et al, 1998). Receptors which have bound growth factor become activated and trigger intracellular signal cascades. These signal

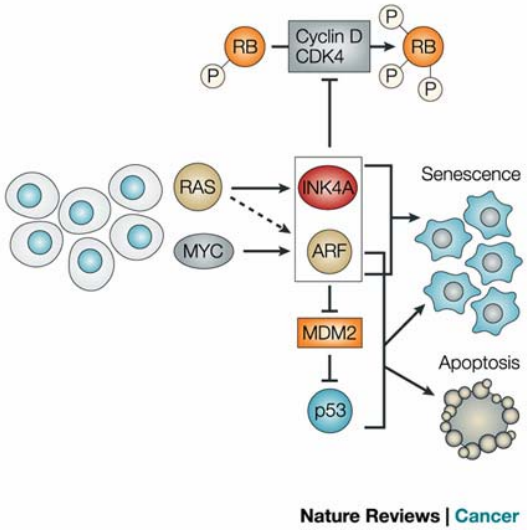
cascades ultimately activate transcription factors, which facilitate expression of genes promoting cell cycle progression. The cell then proceeds to complete cell division. Because these signaling pathways "tell" cells when to divide, an activating mutation of any one component(s) can result in unregulated cell proliferation.

Ras, a family of pro-growth signaling molecules, is particularly relevant to this thesis. Ras genes encode for small G-proteins, important signal transducing molecules which interact with plasma membrane receptors (Downward, 2003). Constitutively active Ras (formed by mutation) will continually transmit pro-growth signals. About 20% of all human tumors have a mutation in one of the alleles for Ras (Downward, 2003). In these tumors, downstream signaling by Ras contributes to the observed phenotype, including deregulation of cell growth, resistance to programmed cell death, and the ability to induce new blood-vessel formation (Shields et al, 2000).

In primary cells, oncogenic Ras^{V12}, a constitutively active Ras, induces senescence via the p53/pRb tumor suppressor pathways (Sherr and Weber, 2000). Recall that this is an example of an activated oncogene (Ras^{V12}) triggering a tumor suppressor pathway (p53/pRb) to control pro-growth signals. Interestingly, mouse embryonic fibroblasts (MEFs) from p53 knockout mice fail to undergo senescence and can be transformed by Ras^{V12} only, suggesting that this tumor suppressor pathway is responsible for senescence activation. A closer look is required to understand exactly how senescence in achieved (Figure 1). Ras^{V12} expression results in an induction of two tumor suppressors $p19^{ARF}$ and $p16^{Ink4a}$ (Serrano et al, 1997). $p19^{ARF}$ stabilizes p53 by promoting degradation of the p53 ubiquitin ligase, MDM2. Increased levels of p53 promote cell cycle arrest and activation of various inhibitors of Cdks, protein complexes which promote cell cycle progression. $p16^{INK4a}$ halts cell cycle progression by blocking pRb repression of E2F, a family of transcription factors promoting G1/S cell cycle progression. Interestingly, pRb can induce cell cycle arrest in p53 deficient

cells, suggesting that pRb may act downstream of p53 (Alexander and Hinds, 2001). Additionally, Rowland et al (2002) showed that E2F repressor complexes are downstream targets of $p19^{ARF}$/p53 senescence. Together, these observations indicate that the p53 and pRb pathways may converge on E2F (Sebastian et al, 2005).

Figure 1: Tumor suppressor response to activated RAS.



Activation of oncogenic RAS causes induction of $p19^{ARF}$ and $p16^{Ink4a}$. $p19^{ARF}$ blocks the activity of MDM2, resulting in activation of p53. $p16^{Ink4a}$ blocks cyclin dependent kinases from phosphorylating pRb. This "fail-safe" response can result in cellular apoptosis or senescence. (Borrowed from Schmitt, 2003).

Bright

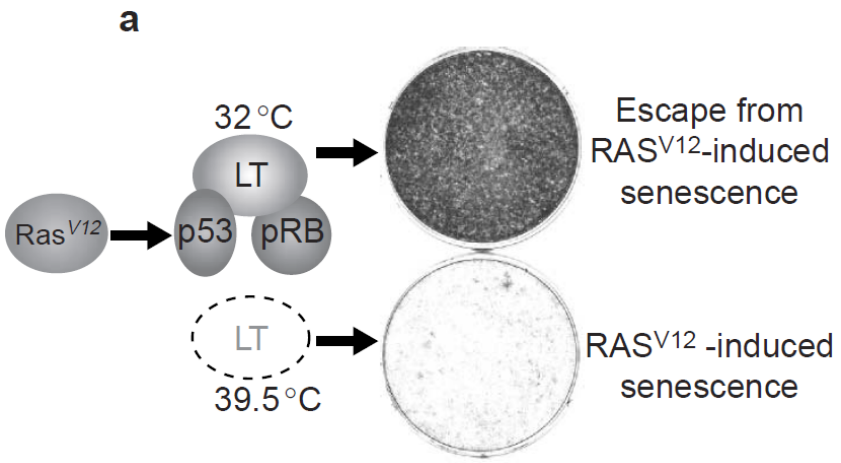
Our protein of interest is Bright (B-cell regulator of immunoglobulin heavy chain transcription; also known as Arid3a), a 70-kDa protein expressed in mouse B-cells. Bright was first discovered as a member of a protein complex binding to regulatory DNA (Webb et al., 1991; Herrscher et al., 1995). It is now known that Bright can upregulate transcription of the immunoglobulin heavy chain (IgH) in mature B-cell lines (Herrscher et al., 1995; Wang et al., 1999). Bright contains a DNA binding domain (ARID, or A-T rich interacting

domain), a trans-activation domain and a multimerization domain (REKLES domain). The ARID DNA-binding domain mediates Bright binding to A-T rich sequences on specific immunoglobulin heavy chain regulatory DNA (Herrscher et al, 1995). It is in this manner that Bright activates IgH transcription. The REKLES domain is required for Bright nuclear import/export and is important in regulating the multimerization of Bright as a homo-tetramer or hetero-tetramer with its paralogue Bdp (Kim et al, 2006; Kim et al, 2007). Additionally, Bright undergoes a cell-cycle dependent shuttling event between the nucleus and cytoplasm (Kim and Tucker, 2006). This shuttling event is essential to Bright's activity. Bright is also post-transcriptionally modified by SUMO (small ubiquitin-related modifier). SUMO is a covalently attached protein modifier which has been shown to alter protein localization (Friedlander and Melchior, 2007).

Human version of Bright is a proto-oncogene

As explained above, activated Ras^{V12} triggers the pRb/p53 tumor suppressor pathways to induce senescence. Therefore, genes which can rescue, or overcome, Ras^{V12} induced senescence must have oncogenic properties. Peeper et al (2002) conducted a retroviral complementary DNA screen in an effort to identify genes that could rescue Ras^{V12} induced senescence in mouse embryonic fibroblasts (MEFs). To reduce background in the screen, Peeper et al (2002) created a unique cell line, called BTR (Figure 2). These cells are also used in our study of Bright. BTR cells express both activated Ras^{V12} and a temperature-sensitive mutant of the simian virus 40 (SV40) Large T antigen, which can induce continuous cell proliferation (Lee et al, 1995). At the permissive temperature (32°), cells rapidly proliferate since the SV40 Large T antigen is active, allowing escape from Ras^{V12} induced senescence. At the non-permissive temperature (39.5°), cells undergo premature senescence

Figure 2: The BTR cell system.

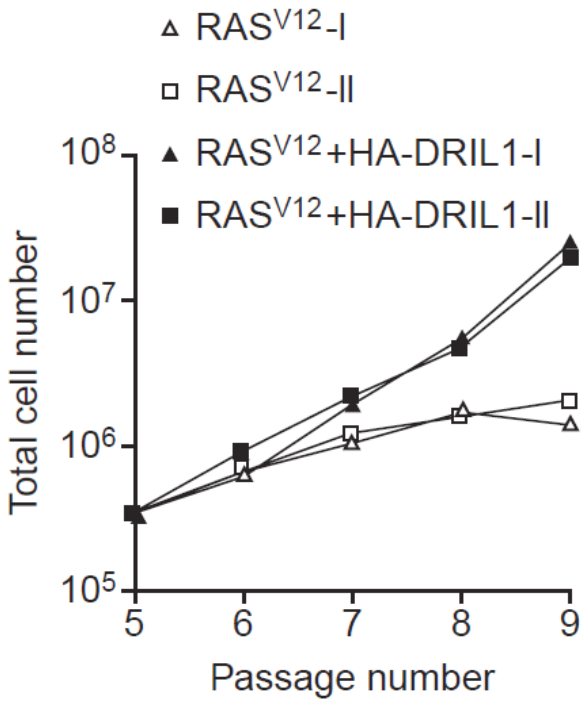


At the permissive temperature (32°C) the SV40 Large T antigen is active, causing BTR cells to proliferate. At the non-permissive temperature (39.5°C) the SV40 Large T antigen is inactive, causing BTR cells to become senescent due to RAS^{V12} expression. (Borrowed from Peeper et al, 2002).

since the SV40 Large T antigen is inactive. Peeper et al found that hDril1, the human orthologue of Bright, can bypass Ras^{V12} senescence at the non-permissive temperature (Figure 3). In fact, hDril1 allows escape from spontaneous senescence in MEFs and can induce tumor formation in nude mice (Peeper et al, 2002). These observations suggest that hDril1 must be interfering with the growth-arrest signals produced by the pRb/p53 tumor suppressor pathways. Peeper et al conducted additional experiments to identify what components of the pathway were being disrupted by hDril1. As expected, Ras^{V12} expression led to an induction of $p19^{ARF}$, $p16^{INK4a}$ and p53. However, co-expression of Ras^{V12} and hDril1 did not interfere with the level of these tumor suppressors as compared to Ras^{V12} expression alone. Thus, it is suggested that hDril1 rescues senescence downstream or independent of $p19^{ARF}$, $p16^{INK4a}$ and p53. The second important result by Peeper et al is that hDril1 senescence rescue is correlated with an induction of CYCLIN E, an important cell

cycle regulator which shuts off pRb by activating its hyperphosphorylation by Cdc2. Overexpression of CYCLIN E is sufficient to allow escape from senescence. Additionally, the CYCLIN E promoter is controlled by the transcription factor E2F1. Therefore, hDril1 may interfere with the tumor suppressor pathway at the level of pRb-E2F1. We investigate this possibility in the results section.

Figure 3: Human Bright, hDril1, can transform senescent cells.



Primary MEFs were retrovirally infected with Ras^{V12} or co-infected with Ras^{V12} and hDril1. As the proliferation curves indicate, hDril1 can transform Ras^{V12}-senescent cells. (Figure borrowed from Peeper et al, 2002).

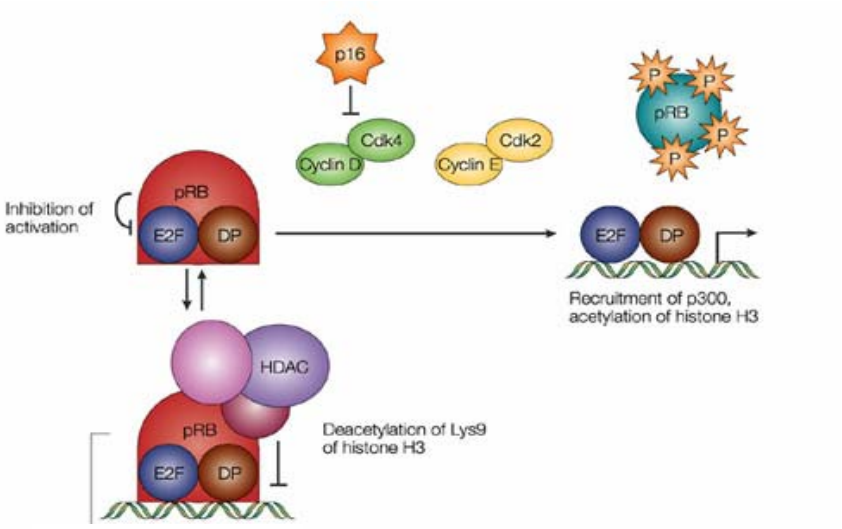
E2F1, pRb and cell cycle regulation:

The balance of positive and negative growth regulatory signals in cells ultimately acts on the E2F transcription factor family, referred to as the "master regulators" of the cell cycle. E2F

controls the transcription of many genes needed to drive the cell cycle, including cell cycle regulators (i.e. cyclins, Cdc2, pRb), enzymes needed in nucleotide production (dihydrofolate reductase, thymidine kinase) and components needed for DNA replication (ORC1) (Trimarchi and Lees, 2002). David Johnson and co-workers found that overexpression of E2F1 complementary DNA was sufficient to activate DNA synthesis in cells that would have undergone growth-arrest (Johnson et al, 1993). Thus, overexpression of E2F1 allows G1/S phase transition. This finding has been supported by other studies (Shan and Lee, 1994; Kowalik et al, 1995). Furthermore, *in vivo* studies have shown that E2F1 overexpressing mice in conjunction with Ras expression can induce tumor development in mice (Pierce et al, 1998).

The model for E2F1 regulation is of particular relevance to this thesis (Figure 4).

Figure 4: Mechanism of E2F1 regulation by pRb



During early G1, pRb is hypophosphorylated and complexes with E2F1-DP. The pRb/E2F1-DP complex then associates with the E2F1 promoter and recruits class I histone deacetylase (HDAC1) which repress transcription. When the cell receives pro-growth signals, cyclin dependent kinases become active and phosphorylate pRb. This releases E2F1-DP allowing it to activate transcription. (Adapted from Trimarchi and Lees, 2002).

Many studies have made it clear that E2F1 is the target of pRb repression (Hamel et al, 1992; Weintraub et al, 1992; Sellers et al, 1995). The phosphorylation state of pRb determines the activity level of E2F1. During growth-arrest or early G1, pRb is hypophosphorylated and forms a complex with E2F1-DP, the functional heterodimer of E2F1. This complex binds to and inhibits transcription of E2F1-controlled genes. The mechanism of this transcriptional repression has been studied extensively. It is suggested that the pRb/E2F1-DP complex binds to the promoter of E2F1-controlled genes and recruits class I histone deacetylase proteins (HDAC1). HDAC1 represses transcription by removing acetyl groups from histones resulting in the condensation of chromatin and therefore, blocked binding of transcription factors. When the cell is growing, pRb becomes hyperphosphorylated by cyclin-dependent kinases and releases E2F1-DP. CYCLIN E is a member of one of these cyclin-dependent kinases. E2F1-DP can then activate transcription of genes needed for G1/S cell cycle progression. Understanding the model of pRb-E2F1 repression is imperative to understanding the results of this thesis.

Results

There were three main aims addressed in this thesis:

(1) Determine which properties of Bright are necessary for transformation of cells.

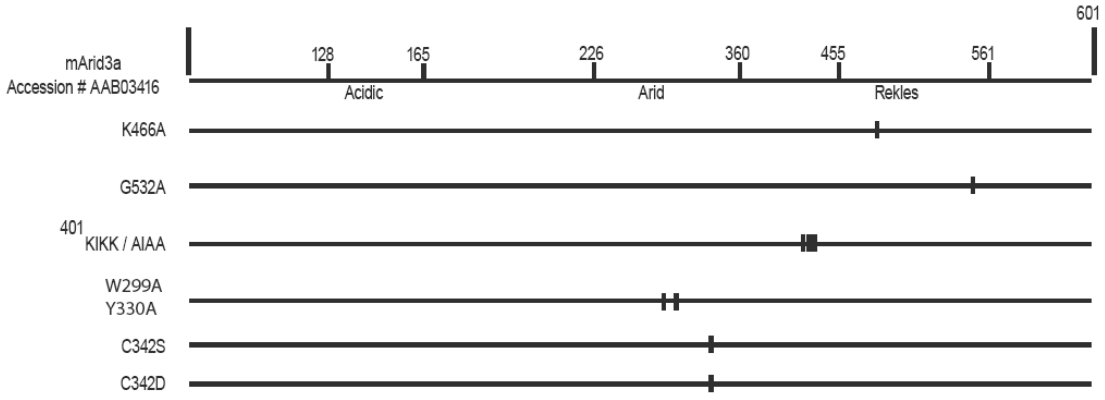
(2) Examine the nature of Bright's interaction with pRb.

(3) Determine if Bright is localized at the E2F1 promoter.

Both wild-type Bright and a sumoylation defective mutant can rescue senescence:

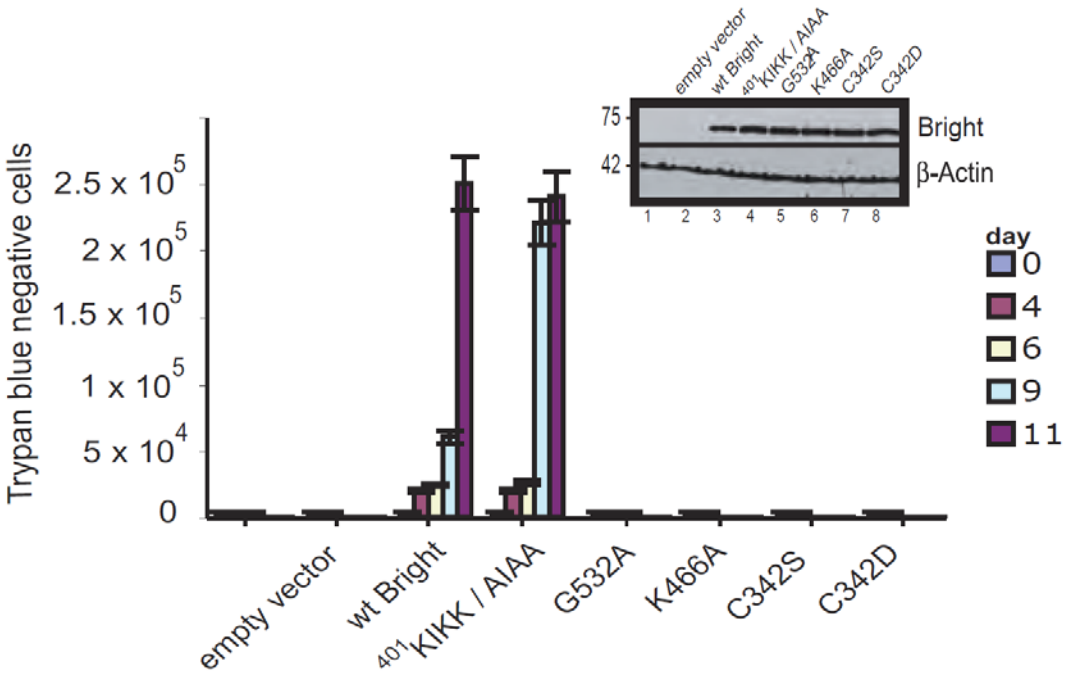
In the first section of this project, we investigated which properties of Bright are necessary for its oncogenic activity. Peeper et al (2002) described the Ras^{V12} senescence rescue ability of hdril1, the human orthologue of Bright. Unpublished data in the Tucker lab has shown that Bright can rescue Ras^{V12} senescence in a manner similar to hdril1 (i.e. downstream of $p53/p19^{ARF}$, correlated with induction of E2F1 and CYCLIN E). We

Figure 5: Schematic of Bright indicating positions of substitution mutations.



Bright point mutants were obtained from Kim and Tucker (2006). K466A is cytoplasm restricted. G532A is nucleus restricted. ^{401}KIKK/AIAA is a sumoylation defective mutant. W299A/Y330A is a true dominant negative form of Bright. C342S and C342D are conservative and non-conservative point mutants of the single conserved cysteine residue in the ARID domain, respectively.

Figure 6: Mouse embryonic fibroblasts are rescued from senescence by Bright and the Bright sumoylation defective mutant ^{401}KIKK/AIAA.



Primary MEFs were infected with the indicated forms of Bright. Trypan blue negative cells were counted. MEFs infected with wild-type Bright and the sumoylation-defective mutant ^{401}KIKK/AIAA show rapid cell proliferation beyond the normal senescence point of these cells, at passage 3.

examined the senescence-rescue ability of five Bright point-mutants made by Kim and Tucker (2006) (Figure 5). The mutant K466A contains a defective nuclear localization sequence and is therefore only present in the cytoplasm. G532A is a nuclear export sequence point mutant and is nucleus restricted. ^{401}KIKK/AIAA is unable to be post-transcriptionally modified by the addition of a sumoylation tag. C342S and C342D are conservative and non-conservative substitution mutants for the single conserved cysteine residue in the ARID DNA binding domain. C342 mutation does not interfere with DNA binding or transactivational properties of Bright (Schmidt et al, 2009).

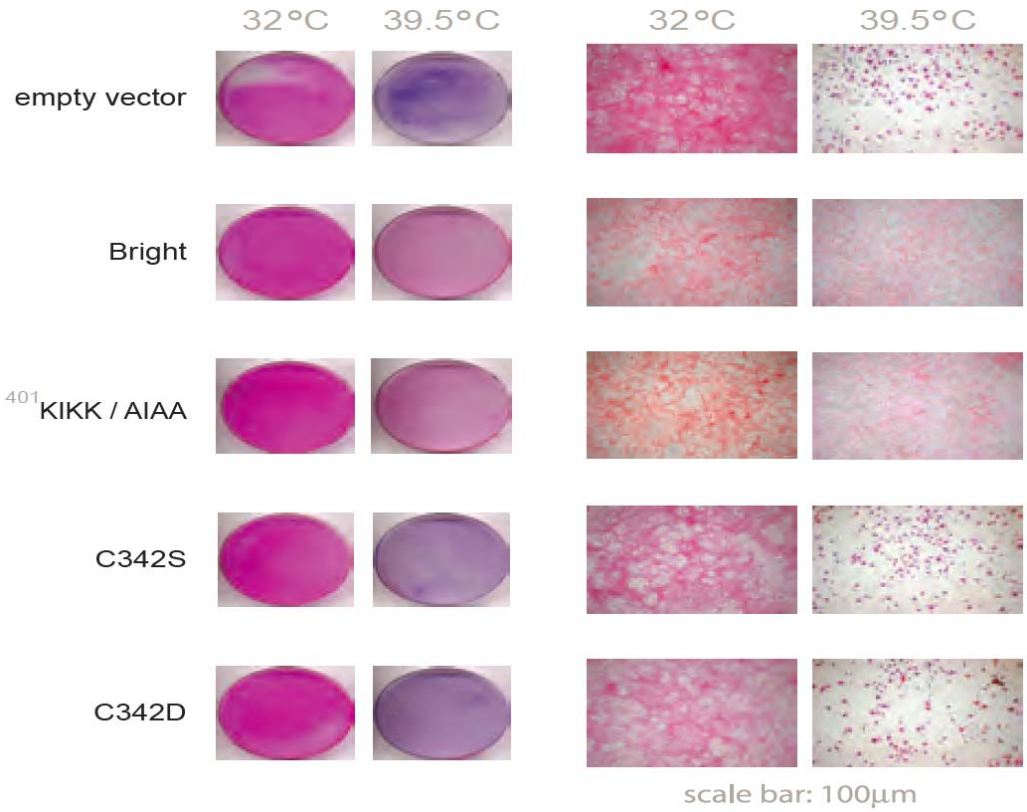
The vector pVxy was used to construct retroviruses containing wild-type and mutant forms of Bright. Mouse embryonic fibroblasts were infected at passage 3. Two days after

infection, cells were grown in the presence of puromycine and the number of trypan blue cells was counted. Our results indicate that only wild-type Bright and the sumoylation defective mutant ^{401}KIKK/AIAA can rescue Ras^{V12} induced senescence in MEFs (Figure 6). This is supported by significantly higher numbers of viable cells counted at day 11 for wild-type Bright and ^{401}KIKK/AIAA compared to the other Bright mutants tested (G532A, K466A, C342S and C342D). The empty vector culture shows extremely low levels of trypan blue-included cells, indicating a low amount of background. Expression of Bright was consistent in all mutant cell lines as indicated by the western blot. The sumoylation defective mutant, ^{401}KIKK/AIAA, appears to induce cell proliferation earlier than wild-type Bright as indicated by higher levels of trypan blue inclusion at day 9 for ^{401}KIKK/AIAA compared to wild-type Bright.

To confirm our results, we stained for senescence associated β-galactosidase activity of BTR cells infected with wild-type and mutant forms of Bright. BTR cells were first grown at the permissive temperature (32°). After two days, cells were shifted to the non-permissive temperature (39.5°) resulting in the inactivation of the SV40 Large T antigen. Senescence was measured by staining cells at acidic pH for β-galactosidase activity (Dimri et al. 1995). Viable cells were measured by their ability to counterstain with Eosin. This results in blue colonies, which are senescent, and red colonies, which are actively proliferating. In agreement with our earlier results, wild-type Bright and the sumoylation defective mutant ^{401}KIKK/AIAA rescued Ras^{V12} senescence at the non-permissive temperature (Figure 7). The lack of contact inhibition (ie, cell density) observed in both cultures suggests that these senescent fibroblasts have undergone transformation, although this conclusion has formally not been confirmed. The empty vector culture served as a negative control and did not rescue senescence. The cysteine mutants, C342S and C342D, also did not rescue senescence. This

suggests that Bright's conserved cysteine in the ARID domain confers a property necessary for Ras^{V12} senescence rescue.

Figure 7: Wild-type Bright and ^{401}KIKK/AIAA can rescue RAS^{V12}-induced senescence in BTR cells.

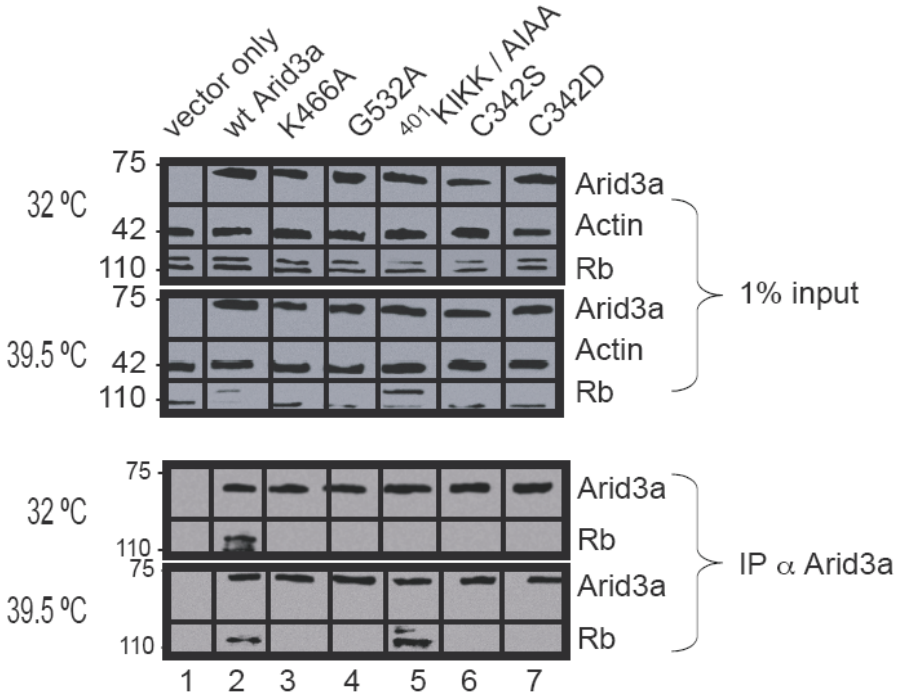


BTR cells were infected with retroviruses encoding the indicated forms of Bright. Cells were grown at the permissive temperature (32 ºC) for three days followed by a shift to the non-permissive temperature (39.5 ºC) for ten days. Cultures were stained for senescence-associated β-galactosidase activity and counterstained with eosin. Photographs of the individual plates are shown. Plates for Bright and ^{401}KIKK/AIAA are red indicating transformation of Ras^{V12} senescent cells. All other Bright mutants tested do not rescue Ras^{V12} senescence and therefore stain blue.

Bright rescuing ability is correlated with hyperphosphorylation of pRb:

In the second phase of this project, we examined a potential association between Bright and pRb. Bright appears to be involved in pRb-E2F1 regulation based on the results of Peeper et al (2002) and unpublished data from the Tucker lab. Investigating a relationship between Bright and pRb may help explain our finding that Bright rescues Ras induced senescence in the previous section. We assayed for pRb levels and phosphorylation status in BTR cells containing wild-type and mutant forms of Bright. Cells were lysed, and then ~50-80 μg of

Figure 8: Bright/Arid3a senescence rescue is correlated with the hyperphosphorylation of pRb.



Btr cells expressing different Bright/Arid3a point mutants were grown at the permissive temperature (32 ºC) for three days followed by a shift to the non-permissive temperature (39.5 ºC) for ten days. Whole cell lysates were prepared and 0.1% of the input was probed with antibodies against Bright, Actin and Rb. Immunoprecipitation of Bright was performed using pre-cleared extract and anti-Bright antiserum. Western analysis indicates that wild-type Bright and ^{401}KIKK/AIAA, forms of Bright which can rescue senescence, have increased levels of hyperphosphorylated pRb at the non-permissive temperature (39.5 ºC).

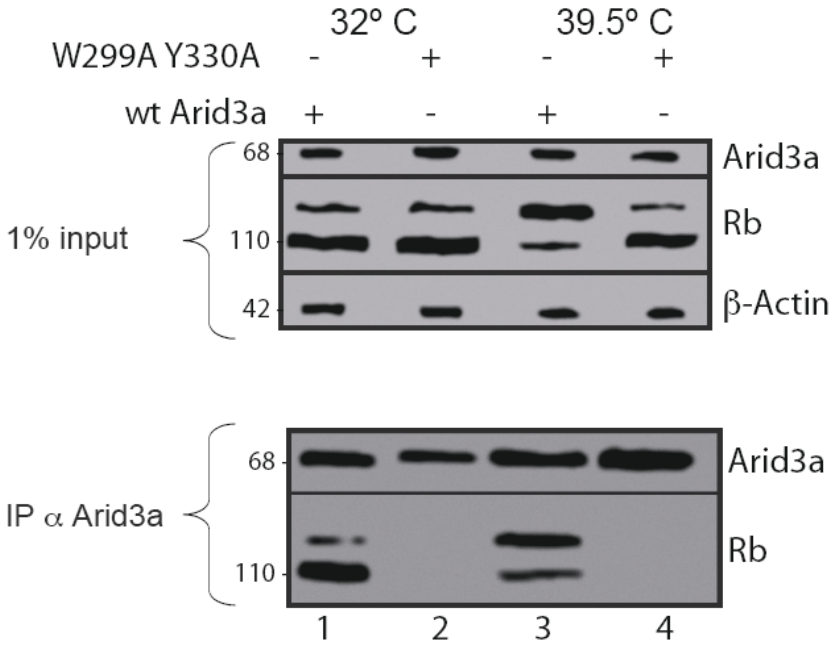
extracted cell proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred from the gel to a nitrocellulose filter and probed using the appropriate antibodies (Figure 8). To ensure that equivalent amounts of extract were loaded onto the SDS-PAGE gel, actin (a ubiquitous protein whose levels are not affected by cell cycle alteration) was used as a loading control. The pRb antibody used in these experiments recognizes both the hypophosphorylated and the hyperphosphorylated forms of pRb. Hypophosphorylated pRb runs slightly faster on the gel than hyperphosphorylated pRb due to differences in charge and conformation. Input lanes show that at the permissive temperature (32°), all BTR cells contain roughly equal levels of hyperphosphorylated and hypophosphorylated pRB (if not slightly more hypophosphorylated pRb). However, at the non-permissive temperature (39.5°), BTR cells expressing wild-type Bright and ^{401}KIKK/AIAA contain increased levels of the hyperphosphorylated form of pRb. Combined with our previous results, this suggests that Bright's Ras^{V12} senescence rescuing ability is correlated with an increase in the level of hyperphosphorylated Bright.

Bright associates with hyperphosphorylated pRb during Ras^{V12} senescence rescue:

Next, we tested for an association between Bright and pRb. Bright was immunoprecipitated out of BTR cell lysates using α-Bright antibody and product was run on a SDS-PAGE gel. The western blot shows that Bright can be found complexed with pRb (Figure 8). Only wild-type Bright interacts with pRb at the permissive temperature (32°) but both wild-type Bright and the ^{401}KIKK/AIAA mutant interact with pRb at the non-permissive temperature (39.5°). Interestingly, none of the other Bright mutants were complexed with pRb. To further test this hypothesis, we examined an additional ARID domain mutant in which residues essential to Bright DNA binding (W299 and Y330) had been changed. Under

conditions in which better resolution of the bands were achieved (Figure 9), we observed clearly that wild-type Bright is predominately complexed with hypophosphorylated pRb at 32° and hyperphosphorylated pRb at 39.5°. Collectively, our results indicate that Bright's association with pRb may be necessary for its function as a proto-oncogene. Since these immunoprecipitation assays cannot verify direct protein-protein interaction, we cannot distinguish whether Bright directly binds to pRb or binds through association of an additional protein(s). Further experiments are needed to resolve these possibilities.

Figure 9: Bright/Arid3a is complexed with hyperphophorylated pRb during senescence rescue.



Western analysis indicates that wild-type Bright is predominantly complexed with hyperphosphorylated pRb at the non-permissive temperature (39.5 ºC). In immortal BTR cells, wild-type Bright is complexed with hypophorphorylated pRb. W299A/Y330A, the dominant negative Bright mutant, cannot be found complexed with pRb.

Bright is recruited to the E2F1 promoter in BTR cells:

There are several lines of evidence that suggest that Bright may be involved in E2F1 regulation. Peeper et al (2002) found that hDril1 rescues Ras^{V12} senescence downstream of $p53/p19^{ARF}$, implying that hDril1 may bypass the tumor suppressor pathway at the level of E2F1. Second, it has been shown that hDril1 can interact with E2F1 (Suzuki et al, 1998). Also, unpublished data in the Tucker lab shows that Bright is complexed with both E2F1 and pRb. To address the third aim of this project, we investigated a possible association between Bright and the E2F1 promoter.

To understand the results of these experiments, it is important to revisit the model of E2F1 gene regulation described in the background section. During senescence (G0/G1 of the cell cycle), hypophosphorylated pRb forms a complex with E2F1-DP, the functional heterodimer of E2F1. This complex binds to the promoter of E2F1 and recruits class I histone deacetylase proteins (HDAC1), which repress transcription. When the cell begins to divide (S phase of the cell cycle), pRb becomes hyperphosphorylated by cyclin-dependent kinases and releases E2F1-DP. E2F1-DP can then activate transcription of E2F1 (Johnson et al, 1994). The results of Peeper et al (2002) imply that hdril1 may be directly involved in E2F1 transcription regulation by pRb. Since Bright can be complexed with pRb and can bind to E2F1, we hypothesized that Bright may be recruited to the promoter of E2F1. This could help explain the observation that Bright induces E2F1 transcription function. Therefore, we assayed for the presence of Bright on the E2F1 promoter region using a molecular biologic technique called chromatin immunoprecipitation (ChIP).

ChIP is used to determine if a protein of interest is localized to a specific region of DNA in the living cell. Cells are first treated with formaldehyde to cross-link DNA binding proteins to chromatin. Following cross-linking, cells are lysed and DNA is sheared through sonication. Protein-DNA complexes are immunoprecipitated using an antibody against the

protein of interest. A sample of this product is run on a SDS-PAGE gel and analyzed by Western blot. The remaining sample is reversed cross-linked and treated with proteinase K to degrade all proteins. The DNA is purified and identified by using polymerase chain reaction (PCR). For the PCR, three primer pairs were designed, two spanning regions outside the E2F1 binding site within the E2F1 promoter region (serving as negative controls), and one spanning the promoter region itself (Figure 10). Primer sequences can be found in the Methods section.

Figure 10: Diagram of ChIP primer landing regions.

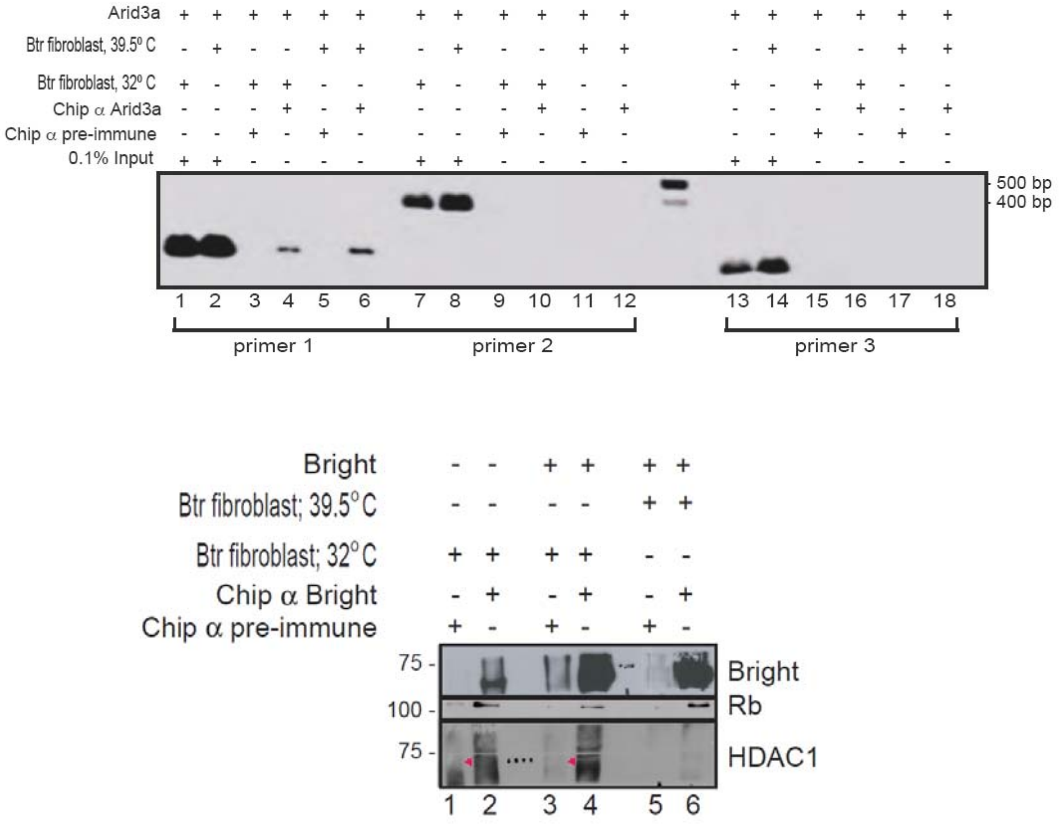
Primer pair 1 spans the E2F1 promoter. Primer pair 3 is far upstream and primer pair 2 is located within the E2F1 coding segments (serving as negative controls for our ChIP experiment).

The first step was to optimize conditions for the ChIP procedure based on our cell line and PCR primers of choice. We established sonication conditions to shear cross-linked DNA to 200-1000 base pairs in length in BTR cells. Additionally, we established PCR conditions to enhance specificity of E2F1 promoter DNA amplification. We confirmed our ChIP optimization by assaying for an association between Bright and purified DNA from Bright overexpressing NIH/3T3 cell lysates (data not shown). Following cross-linking and sonication, protein-DNA complexes were immunoprecipitated using anti-Bright antibody. Western analysis of this sample indicated the presence of Bright. After reverse cross-linking

and DNA purification, we were able to successfully amplify DNA using Primer pair 1, which spans the E2F1 promoter. There was no amplification product for Primer pair 2 or for Primer pair 3, confirming that our reaction conditions did not cause nonspecific binding of Bright to chromatin. With our ChIP protocol optimized, we proceeded to perform a ChIP assay against Bright in BTR cells.

Wild-type Bright infected BTR cells were cross-linked and lysed. Lysates were

Figure 11: Bright is recruited to the E2F1 promoter. Senescence rescue is correlated with the loss of HDAC1 at the E2F1 promoter.

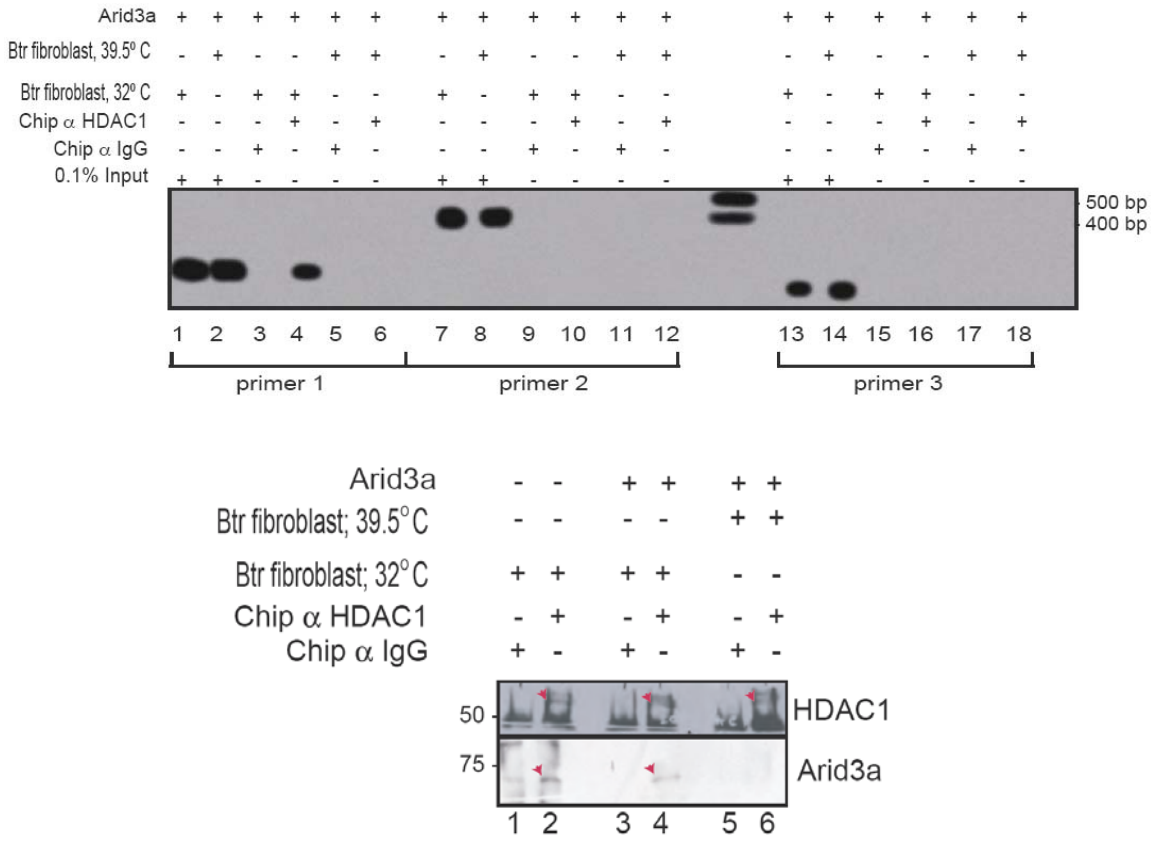


BTR cells were infected with Bright/Arid3a encoding viruses as indicated and grown at the permissive temperature for two days, followed by shift to the non-permissive temperature for ten days. PCR and Southern hybridization of immunoprecipitation DNA product is shown in the top panel. Western analysis of the immunoprecipitated DNA-protein complexes is shown in the bottom panel. Bright is found localized at the E2F1 promoter. At the permissive temperature, Bright is found complexed with pRb and HDAC1 at the E2F1 promoter. At the non-permissive temperature, HDAC1 is lost at the E2F1 promoter.

sonicated and immunoprecipitation was performed using α-Bright antibody. A small portion of the IP product was analyzed by Western blot. The remaining sample was reverse cross-linked, treated with proteinase K and DNA was purified. DNA was amplified by PCR and product was run on a DNA agarose gel followed by transfer to a nitrocellulose membrane and southern hybridization using radiolabeled primers. Signal in lane 4 and 6 of the southern blot shows that wild-type Bright is localized to the E2F1 promoter region at both the permissive temperature (32°) and the non-permissive temperature (39.5°; Figure 11). ChIP using α-preimmune serum served as a negative control confirming specificity of the α-Bright antibody. Primer 2 and Primer 3 produced negative results in the experimental lanes supporting the conclusion that Bright is localized to the E2F1 promoter. This also verifies that Bright's localization on chromatin is not an artifact of the ChIP protocol. Additionally, the positive signal in the input lanes indicates that total chromatin was successfully isolated from the BTR cells. Assuming equal loading, there appears to be more Bright located on the E2F1 promoter at 39.5° than 32° in BTR cells. Western analysis of the immunoprecipitation product is shown in Figure 11. Bright localized to the E2F1 promoter is associated with HDAC1 and pRb at the permissive temperature (32°). However, at the non-permissive temperature (39.5°), Bright localized to the E2F1 promoter is associated with pRb only and not HDAC1.

To confirm our results, we performed another ChIP assay using α-HDAC1 antibody for the immunoprecipitation step (Figure 12). Southern hybridization indicates that HDAC1 can be found at the E2F1 promoter at the permissive temperature (32°) but not at the non-permissive temperature (39.5°). The signal in lane 4 of the α-Bright Western confirms that Bright is found associated with HDAC1 on the E2F1 promoter at the permissive temperature.

Figure 12: Reciprocal ChIP assay verifies that HDAC1 association with the E2F1 promoter and Bright is lost during senescence rescue.



ChIP was performed using α-HDAC1 antibody. HDAC1 is complexed with Bright and recruited to the E2F1 promoter at the permissive temperature (lane 4 bottom panel; lane 4 top panel). Consistent with the results of the first ChIP experiment, HDAC1 does not associate with Bright or the E2F1 promoter at the non-permissive temperature (lane 6 bottom panel; lane 6 top panel).

The lack of signal in lane 6 of the α-Bright Western supports our earlier result that HDAC1 does not associate with Bright on chromatin at the non-permissive temperature.

In both of the ChIP α-Bright Westerns, we noticed signal in Bright negative lanes (lanes 1 and 2 of Figure 11; lanes 1 and 2 of Figure 12). There are two possible origins of these signals. First, they could be the result of nonspecific binding of the α-Bright antibody to an unknown protein. This seems likely since the recognized band runs lower than 74 kDa, the size of Bright. The second possible explanation is that there is endogenous expression of Bright in BTR cells. The Tucker lab has found that Bright expression is not tightly B-cell

restricted and there is trace endogenous expression in some cell lines, including fibroblasts. Since BTR cells are made from fibroblast cells, these bands could be endogenous Bright. We were unable to further investigate this due to time constraints.

These results suggest that Bright senescence rescue is correlated with the loss of HDAC1 at the E2F1 promoter. While our ChIP assay has found an association between Bright and the E2F1 promoter, we cannot confirm direct binding of Bright to DNA. EMSA assays have the capability of detecting direct binding of proteins to DNA. Our preliminary EMSA results are consistent with the direct binding of Bright at the E2F1 promoter (data not shown).

Discussion

The goal of this thesis was to investigate the oncogeneic properties of Bright. First, we tested five mutant forms of Bright to determine which ones retained the ability to transform cells. We assayed for both natural senescence rescue and Ras^{V12} senescence rescue. Only wild-type Bright and a sumoylation defective mutant were capable of bypassing senescence. Next, we looked for changes in pRb levels in cells transformed by Bright and assayed for an association between Bright and pRb. Bright immortalization was found to be correlated with increased levels of hyperphosphorylated pRb, indicating G1/S progression. Additionally, Bright was found to complex predominantly with hyperphosphorylated pRb during senescence bypass. Finally, we used chromatin immunoprecipitation to show that Bright is recruited to the E2F1 promoter. Our results extend the findings of Peeper et al (2002) by identifying a potential mechanism for Bright's oncogenic properties: Bright disrupts pRb/E2F1 complexes from repressing transcription of the E2F1 promoter. As a result, E2F1 transcription is de-repressed allowing transcription of genes necessary for G1/S cell cycle progression. While more experiments are needed to clarify the details of our proposed model, our results provide a good starting point for future work.

Our first aim was to determine which properties of Bright are necessary to transform cells. We conducted cell proliferation assays to determine if various mutant forms of Bright could rescue natural senescence in primary MEFs. We confirmed our results by using senescence associated β-galactosidase staining to test for rescue of Ras^{V12} induced senescence in BTR cells. Interestingly, the sumoylation defective mutant, ^{401}KIKK/AIAA, rescued senescence along with wild-type Bright. This suggests that sumoylation is not necessary for Bright to function as a proto-oncogene. All other mutant forms of Bright tested did not transform cells. In the context of our proposed model, it is expected that Bright

containing a defective nuclear localization sequence should not rescue senescence. Without a functional NLS, Bright cannot enter the nucleus to de-repress the E2F1 promoter. But, why does the nucleus-restricted Bright mutant (G532A) not rescue senescence? A likely explanation is that the nuclear export sequence mutation makes Bright unable to interact with pRb through its ARID domain. In fact, this is probably why the cysteine substitution mutants also did not rescue senescence. Our immunoprecipitation experiments support this idea since these mutant forms of Bright do not associate with pRb (Figure 8). Overall, it appears that Bright must be transcriptionally competent and able to associate with pRb to rescue senescence.

The second phase of this project examined the association between Bright and pRb to investigate the possibility that Bright may be involved in pRb-E2F1 regulation. Western analysis showed that Bright immortalization is correlated with the hyperphosphorylation of pRb. This is an indicator of cell proliferation since pRb normally becomes hyperphosphorylated by cyclin-dependent kinases during G1/S transition. Our immunoprecipitation assays revealed that Bright predominantly complexes with the hyperphosphorylated form of pRb during senescence rescue. The observation that Bright can associate with pRb lends support to our model of senescence bypass. Bright may associate with pRb/E2F1-DP complexes to interfere with their normal repression of the E2F1 promoter. This seems likely since our data suggest that Bright is complexed with the pRb that is regulating E2F1.

Our immunoprecipitation assays were unable to confirm direct binding of Bright to pRb. Proteins typically bind to pRb through the LXCXE motif (Dahiya et al, 2000). However, many cell cycle regulators, including E2F1, have been shown to bind to the COOH-terminal region of pRb. Bright's paralogue, Bdp, binds pRb in this fashion via its ARID domain (Numata et al, 1999). The high degree of similarity between Bright and Bdp

(89% identical) makes it likely that Bright can also bind pRb via its ARID domain. Thus, mutation of the conserved cysteine (in Bright point mutants C342S and C342D) probably results in the inability of the ARID domain to interact with pRb. It is important to note that all the mutants that failed to rescue senescence, including the dominant negative mutant, also failed to complex with pRb. This suggests that association with pRb is essential for Bright to function as a proto-oncogene, a conclusion consistent with our model of Bright senescence rescue.

In the last set of experiments, we assayed for an association between Bright and the E2F1 promoter. Unpublished data in the Tucker lab indicated that Bright rescues senescence in the same manner as hDril1, downstream of $p19^{ARF}$, $p16^{INK4a}$ and p53. Bright immortalization also leads to the induction of E2F1 and CYCLIN E, a cell cycle regulator whose transcription is activated by E2F1. Since E2F1 regulation appears to be affected during transformation, we hypothesized that Bright may affect the $p19^{ARF}$/p53 tumor suppressor pathway at the level of pRb/E2F1. Our results are consistent with our hypothesis. Bright appears to transform cells by disrupting pRb/E2F1-DP repressive complexes on the E2F1 promoter. This conclusion is based on the observation that HDAC1 is displaced from the E2F1 promoter and fails to associate with Bright/pRb complexes during senescence rescue (Figures 11 and 12).

There are several possible mechanisms of Bright's actions. One possibility is that Bright may sequester hypophosphorylated pRb in the cytoplasm during transformation. Presumably, Bright would bind hypophosphorylated pRb and then shuttle to the cytoplasm during G1, stopping hypophosphorylated pRb from forming pRb-E2F1 repressive complexes in the nucleus. Consistent with this view is the finding that Bright is predominantly localized in the cytoplasm during G1 and shuttles to the nucleus during S phase (Kim and Tucker, 2006). Bright shuttling would stop pRb from forming pRb/E2F1-DP repressive complexes in

the nucleus. This model, however, fails to explain our observation that pRb remains associated with the E2F1 promoter during senescence rescue (Figure 11, Western lane 6).

Alternatively, Bright may block the recruitment HDAC1 to the E2F1 promoter. In this mechanism, Bright first associates with hypophosphorylated pRb/E2F1-DP repressive complexes. Next, the Bright/pRb/E2F1-DP complex enters the nucleus and is recruited to the E2F1 promoter. For an unknown reason or by a mechanism yet to be determined, Bright then blocks pRb/E2F1-DP complexes from recruiting HDAC1 to the E2F1 promoter. This possibility is supported by our ChIP data showing that during immortalization, Bright and pRb maintain association with the E2F1 promoter without the recruitment of HDAC1. A possible signal for Bright to disrupt HDAC1 recruitment could be low levels of nuclear E2F1, since senescent cells have low nuclear E2F1 and Bright has been characterized as an E2F1 binding protein (Suzuki et al, 1998).

While our results help narrow down the general action of Bright, there are several lines of future work that should be pursued. First, more experimentation is needed to understand the interaction between Bright and pRb. Performing i*n vitro* binding assays for Bright and pRb may prove insightful. Additionally, the senescence rescuing ability of Bright mutants which are unable to complex/bind to pRb but retain all other functions should be examined. This would help rule out the possibility that Bright's interaction with pRb is unnecessary for senescence rescue. Another issue worthy of future experimentation is the action of Bright at the E2F1 promoter. Electron mobility shift assays could confirm direct binding of Bright to E2F1 promoter DNA. If Bright does bind directly, then it would be valuable to examine the senescence rescuing ability of Bright mutants which are unable to bind chromatin but still interact with pRb and E2F1. Finally, Bright's potential for tumor formation *in vivo* should be studied. There may be species specific differences in the ability

of Bright vs. hDril1 to form tumors in mice. Additionally, experiments studying the phenotype of Bright overexpressing transgenic mice may prove insightful.

Methods

Preparation of MEFs, cell culture and retroviral infection: Organs and head were removed from a 15 day old mouse embryo, and the remaining tissue was washed in phosphate buffered saline (PBS) and minced. After a second PBS wash, the tissue was incubated with 100 µl trypsin/EDTA (Gibco) on ice for 12 hr. The tissue was incubated with 100 µl trypsin/EDTA at 37 °C for 30 min, dissociated in complete medium, and transferred to a 100-mm dish. MEFs were maintained in DMEM (Gibco) supplemented with 10% FBS (PAA Laboratories) and 0.1 mM β-mercapto ethanol. These MEFs were designated as passage 1 and were maintained in the same media.

Phoenix packaging cells were used to generate ecotropic retroviruses as described (Serrano et al., 1997). MEFs were infected with filtered (0.45 µm) viral supernatant, supplemented with 4-8 µg/ml polybrene. In general, a single infection round of 6 hrs was sufficient to infect at least 90% of the population.

Btr cells (Peeper et al., 2002) were maintained at 32 ºC and infected using viruses as described above.

Proliferation curves: For proliferation curves, passage 3 MEFs were infected with retroviral vectors carrying selectable markers. At 1-2 days post infection, they were selected with puromycin (1-3 µg/ml) for 5-7 days. The number of trypan blue negative cells were counted at the indicated time points.

Senescence-associated β-galactosidase staining: Senescence-associated β -galactosidase activity was detected as previously described (Dimri et al., 1995). Cells were washed once with PBS (pH 7.2), fixed with 1% glutaraldehyde in PBS (pH 7.2) for 30 min at room

temperature, and washed once in PBS (pH 7.2) supplemented with 1mM MgCl2. Cells were then stained in X-gal solution (1mg/ml X-gal, 120μM K3Fe[CN]6, 120μM K4Fe[CN]6, 1mM MgCl2 in PBS at pH 6.0) overnight at 37º C without CO2. Counterstaining with Eosin (Sigma) was performed according to the manufacturer's instruction.

Western blotting, IP and Chip: For Western blotting, cell extracts were prepared in NETN lysis buffer (100 mM NaCl, 0.5% NP-40, 50 mM TRIS-HCl pH 8.0, 1 mM EDTA, supplemented with a protease inhibitor cocktail (Boehringer), assayed for protein concentration, and 50-80 μg of clarified extract was resolved on SDS-PAGE gels, followed by transfer onto nitrocellulose membranes and probing with antisera, as described in Kim and Tucker (2006). Primary antibodies used for Western blotting were R562 (Abcam) for p19ARF, ab9113 (Abcam) for the v5 tag, ab28305 (Abcam) for E1A, 3965 (Cell Signaling) for ras, Ab7 (Calbiochem) for p53, and from Santa Cruz: M-156 for p16INK4a, C-19 for p21CIP1, M-20 for Cyclin E, C-22 for Cdk4, H-295 for Cyclin D1 and C-22 for actin. Dr. Julien Sage, Stanford University, kindly provided the anti-Rb antibody (Sage et al., 2000). Affinity-purified, rabbit polyclonal anti-mArid3a generated against full-length bacterially synthesized and purified Arid3a has been described (Herrscher et al., 1995). Enhanced chemoluminescence (Amersham) was used for detection of proteins. Actin and tubulin served as loading controls for all blots. Chip assays were performed using the Upstate Chip Kit (17-295) according to the manufacturer's instructions (Attema et al., 2007).

E2F1 Primer Pair 1:

Invitrogen

3' Primer, Lot# 10336022120836, Sequence: GCTGGAATGGTGTCAGCACAGCG

5' Primer, Lot# 10336022120836, Sequence: TCCAAGAATCATATCCAGTGGCT

E2F1 Primer Pair 2:

Invitrogen

3’ Primer, Lot# 10336022120836, Sequence: AACACTTGCTGCCAGGACTT

5’ Primer, Lot# 10336022120836, Sequence: TGGCTCACAACCACCTGTAA

E2F1 Primer Pair 3:

Invitrogen

3’ Primer, Lot# 10336022120836, Sequence: GTCCCACCCTCCGTCTCC

5’ Primer, Lot# 10336022120836, Sequence: AAAGTCCGGGCCACTTTTAC

References

Alexander K, PW Hinds. 2001. Requirement for p27(KIP1) in retinoblastoma protein-mediated senescence. *Mol Cell Biol.* 21:3616–3631.

Bandara LR, NB La Thangue. 1991. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature.* 351(6326):494–497.

Campisi, J. 2001. Cellular senescence as a tumour-suppressor mechanism. *Trends Cell Biol.* 11, S27−S31

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, JR Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell.* 65(6):1053–1061.

Dahiya A, Gavin MR, Luo RX, DC Dean. 2000. Role of the LXCXE binding site in Rb function. *Mol. Cell Biol.* 20:6799–6805.

Darnell, JE Jr. 2002. Transcription factors as targets for cancer therapy. *Nature Rev. Cancer* 2:740–749.

Dimri G, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* 92:9363-9367.

Downward, J. 2003. Targeting ras signalling pathways in cancer therapy. *Nat Rev Cancer.* 3(1):11-22.

Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes Dev.* 12, 2245–2262.

Evan GI, KH Vousden. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature.* 411:342−348.

Geiss-Friedlander R, Melchior F. 2007. Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol.* 8:947–956

Hahn WC, RA Weinberg. 2002. Modelling the molecular circuitry of cancer. *Nature Rev. Cancer* 2:331–341.

Hamel PA, Gill RM, Phillips RA, BL Gallie. 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. *Mol Cell Biol*. (8):3431–3438.

Hanahan D, RA Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57–70.

Hayflick L. 1965. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res.* 37:614–636

Herrscher RF, Kaplan MH, Lelsz DL, Das C, Scheuermann R, PW Tucker. 1995. The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific trans-activator that describes a new DNA-binding protein family. *Genes Dev.* 9:3067.

Howe A, Aplin AE, Alahari SK, RL Juliano. 1998. Integrin signaling and cell growth control. *Curr Opin Cell Biol.* 10(2):220–231.

Johnson DG, Schwarz JK, Cress WD, JR Nevins. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature.* 365(6444):349–352.

Kim D, and PW Tucker. 2006. A Regulated Nucleocytoplasmic Shuttle Contributes to Bright's Function as a Transcriptional Activator of Immunoglobulin Genes. *Mol. Cell. Biol.* 26:2187-2201.

Knudson, AG. 1971. Mutation and cancer: Statistical study of retinoblastoma. *Proc Natl Acad Sci USA*. 68:820–823.

Kortschak, RD, Reimann H, Zimmer M, Eyre HJ, Saint R, and DE Jenne. 1998. The human dead ringer/bright homolog, DRIL1: cDNA cloning, gene structure, and mapping to D19S886, a marker on 19p13.3 that is strictly linked to the Peutz-Jeghers syndrome. *Genomics.* 51:288-292.

Kowalik TF, DeGregori J, Schwarz JK, Nevins JR. 1995. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J Virol.* 69(4):2491–2500.

Lee GH, Ogawa K, and NR Drinkwater. 1995. Conditional transformation of mouse liver epithelial cells. An *in vitro* model for analysis of genetic events in hepatocarcinogenesis. *Am. J. Pathol.* 147:1811–1822.

Nordling, CO. 1953. A new theory on cancer-inducing mechanism. *Br J Cancer.* 7:68–72.

Numata S, Claudio PP, Dean C, Giordano A, CM Croce. 1999. Bdp, a new member of a family of DNA-binding proteins, associates with the retinoblastoma gene product. Cancer Res. *59:*3741-3747.

Pierce AM, Fischer SM, Conti CJ, and DG Johnson. 1998. Deregulated expression of E2F1 induces hyperplasia and cooperates with *ras* in skin tumor development. *Oncogene*. 16:1267-1276.

Schmitt CA. 2003. Senescence, apoptosis and therapy—cutting the life-lines of cancer. *Nat Rev Cancer.* 3:286–295.

Sebastian T, Malik R, Thomas S, Sage J, PF Johnson. 2005. C/EBPbeta cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. *EMBO J.* 24:3301–3312.

Sellers WR, Rodgers JW, Kaelin WG Jr., DM Livingston. 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc Natl Acad Sci USA.* 92:11544-48.

Serrano M, & MA Blasco. 2001. Putting the stress on senescence. *Curr. Opin. Cell Biol.* 13:748–753.

Serrano M, Lin AW, McCurrach ME, Beach D, SW Lowe. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 88(5)**:**593-602.

Shan B, WH Lee. 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol Cell Biol.* (12):8166–8173.

Shields JM, Pruitt K, McFall A, Shaub A, CJ Der. 2000. Understanding ras: "It ain't over 'til it's over." *Trends Cell Biol.* 10:147-154.

Shirodkar S, Ewen M, DeCaprio JA, Morgan J, Livingston DM, T Chittenden. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell.* 68(1):157–166.

Suzuki M, Okuyama S, Okamoto S, Shirasuna K, Nakajima T, Hachiya T, Nojima H, Sekiya S, and K Oda. 1998. A novel E2F binding protein with Myc-type HLH motif stimulates E2F-dependent transcription by forming a heterodimer. *Oncogene* 17:853-865.

Trimarchi JM, JA Lees. 2002. Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol.* 3:11−20.

Vogelstein, B. & KW Kinzler. 2004. Cancer genes and the pathways they control. *Nature Med.* 10:789–799

Wang Z, Goldstein A, Zong RT, Lin D, Neufeld EJ, Scheuermann RH, PW Tucker. 1999. Cux/CDP homeoprotein is a component of NF-μNR and represses the immunoglobulin heavy chain intronic enhancer by antagonizing the *Bright* transcription activator. *Mol. Cell. Biol.* 19:284.

Webb CF, Das C, Eaton S, Calame K, PW Tucker. 1991. Novel protein-DNA interactions associated with increased immunoglobulin transcription in response to antigen plus interleukin-5. *Mol. Cell. Biol.* 11:5197.

Webb CF, Smith EA, Medina KL, Buchanan KL, Smithson G, S Dou. 1998. Expression of *Bright* at two distinct stages of B lymphocyte development. *J. Immunol.* 160:4747.

Weinberg RA. 1989. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Res.* 49:3713−3721.

Weintraub SJ, Prater CA, DC Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature.* 358(6383):259–261.

Xiao B, Spencer J, Clements A, Ali-Khan N, Mittnacht S, Broceno C, Burghammer M, Perrakis A, Marmorstein R, SJ Gamblin. 2003. Crystal structure of the retinoblastoma tumor suppressor protein bound to E2F and the molecular basis of its regulation. *Proc Natl Acad Sci USA*. 100:2363–8.

Acknowledgements

I owe many thanks to Dr. Christian Schmidt who taught me various laboratory techniques and provided constant encouragement during my growth as a student. Thanks to Chhaya Das and Maya Ghosh for their willingness to help me on a daily basis in the Tucker lab. Finally, I would like to thank Dr. Philip Tucker for his invaluable guidance and support throughout my years in college. He has inspired me to pursue a career in academic medicine so that I can remain immersed in scientific research.